Phylogenetic assessment of culture collection strains of *Thiobacillus thioparus*, and definitive 16S rRNA gene sequences for *T. thioparus*, *T. denitrificans* and *Halothiobacillus neapolitanus*

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Footnotes

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Nucleotide sequence data reported are available in the DDBJ/ EMBL/GenBank databases under the accession numbers HM173629 (*T. thioparus* ATCC 8158^T), HM173630 (*T. thioparus* NCIMB 8370^T), GU967679 (*T. thioparus* DSM 505^T), HM535226 (*T. thioparus* THI 111, JCM 3859^T, NBRC 103402^T), GU967680 (*T. thioparus* DSM 5369), GU967681 (*T. thioparus* DSM 5368), HM173633 (*T. thioparus* ATCC 23645), HM173634 (*T. thioparus* ATCC 23647), HM173635 (*T. thioparus* ATCC 23646), HM535225 (*T. thioparus* THI 115, NBRC 105750), JF416645 (*Halothiobacillus neapolitanus* NCIMB 8539^T), HM173632 (*Halothiobacillus neapolitanus* NCIMB 8454), and HM173631 (*Thermithiobacillus* sp. NCIMB 8349).

Abstract The 16S rRNA gene sequences of 12 strains of *Thiobacillus thioparus* held by different culture collections have been compared. A definitive sequence for the reference type strain (Starkey; ATCC 8158^T) was obtained. The sequences for four examples of the Starkey type strain were essentially identical, confirming their sustained identity after passage through different laboratories. One strain (NCIMB 8454) was reassigned as a strain of *Halothiobacillus neapolitanus* and a second (NCIMB 8349) was a species of *Thermithiobacillus*. These two strains have been renamed in their catalogue by the National Collection of Industrial and Marine Bacteria. The 16S rRNA gene sequence of the type strain of *Halothiobacillus neapolitanus* (NCIMB 8539^T) was determined, and used to confirm the identity of other culture collection strains of this species. The reference sequences for the type strains of *Thiobacillus thioparus* and *Halothiobacillus neapolitanus* have been added to the on-line *List of Prokaryotic Names with Standing in Nomenclature*. Comparison of the 16S rRNA gene sequences available for strains of *Thiobacillus denitrificans* indicated that the sequence for the type strain (NCIMB 9548^T) should always be used as the reference sequence for new and existing isolates.

Keywords *Halothiobacillus neapolitanus, Thermithiobacillus, Thiobacillus X*, *Thiobacillus thioparus*, *Thiobacillus denitrificans*, type strains

Introduction

Sulfur bacteria commonly known as thiobacilli are ubiquitous in the natural environment, and are chemolithithoautotrophic *Proteobacteria* which gain energy from sulfur compound oxidation to fix carbon dioxide for biosynthesis and growth. Some species are halophiles, thermophiles, extreme acidophiles, or facultative anaerobes, and some tolerate high levels of toxic metals (Hutchinson et al. 1966, 1967; Rawlings 2002; Wood and Kelly 1985, 1991). Their activities can be both damaging to the environment, and exploited for biotechnology. Sulfuric acid production from their metabolism can cause extreme damage to concrete structures, and their production of acid mine drainage containing toxic metals can cause severe pollution of water and soils (Evanglou and Zhang 1995; Kelly 2010; Mudd and Patterson 2010; Parker 1945; Parker and Jackson 1965). Mineral leaching by some species has been used for many years for the economic recovery of metals, principally copper, but including uranium, nickel, zinc and gold (Brandl 2008; Chen et al. 2008; Ehrlich and Brierley 1990; Kelly 1985; Rawlings 2002). Denitrifying strains have been used in the bioremediation of nitrate-polluted waters, and both aerobic and anaerobic strains have been used to degrade thiocyanate, and to remove hydrogen sulfide and methylated sulfides from contaminated air streams or natural gas (Aroca et al. 2007; Kanagawa and Kelly 1986; Kanagawa and Mikami 1989; Katayama and Kuraishi 1978; Ramirez et al. 2009; Sublette and Sylvester 1987; Zhang et al. 2009). There is also evidence that some species can underpin chemolithotrophically-driven ecosystems in the absence of photosynthetic energy (Chen et al. 2009). Intensive study of their biochemistry has not yet fully elucidated their mechanism(s) of sulfur

89 compound oxidation, and possible differences among different groups of thiobacilli
90 necessitate precise taxonomic characterization of strains and species.
91 The genus *Thiobacillus* was created by Beijerinck (1904) to comprise two species
92 of obligately chemolithoautotrophic sulfur-oxidizing bacteria: the aerobic
93 *Thiobacillus thioparus* and the facultative denitrifier, *Thiobacillus denitrificans*.
94 Subsequently, numerous other additional species were described, differentiated by
95 colony morphology and a limited number of physiological characteristics (as the
96 standard criteria applied to heterotrophs could not be applied to obligate
97 chemolithotrophs), including variation in the sulfur substrates used and the oxidation
98 products observed. Between 1923-1998, paralleling the first to ninth editions of
99 *Bergey's Manual of Determinative Bacteriology* (1923-1994) and the first edition of
100 *Bergey's Manual of Systematic Bacteriology* (1998), at least 32 species were named,
101 many of which were never validated or were subsequently lost from culture. The first
102 comprehensive attempt to assess the relationships of a number of species used
103 numerical taxonomy based on numerous physiological and cultural properties
104 (Hutchinson et al. 1965, 1966, 1967, 1969). Subsequently, with the advent of 16S
105 rRNA gene sequencing and other diagnostic molecular methods, a number of species
106 were transferred to other existing or new genera, including *Acidiphilium,*
107 *Acidithiobacillus, Halothiobacillus, Paracoccus, Starkeya, Thermithiobacillus* and
108 *Thiomonas* (Battaglia-Brunet et al. 2011; Hiraishi and Imhoff 2005; Katayama et al.
109 2006; Kelly and Wood 1998, 2000a; Kelly et al. 2000, 2005, 2007). The 2nd edition
110 of *Bergey's Manual of Systematic Bacteriology* recognized only three validly named
111 species and one putative species (Kelly et al. 2005). Currently only six distinct
112 species can be accepted on the basis of their 16S rRNA gene sequences, namely *T.*
113 *thioparus, T. denitrificans, T. aquaesulis, T. thiophilus, "T. plumbophilus",* and *"T.*

114 *sajanensis"*. Of these, only Beijerinck's original species, *T. thioparus* and *T.*
115 *denitrificans* (Beijerinck 1904), have been retained through all the editions of
116 *Bergey's Manuals*. The original isolates of those species were lost (L. A. Robertson,
117 Delft, personal communication), and no culture collection reference strains were cited
118 in the 8th edition of *Bergey's Manual* (Vishniac 1974). The type strain of the type
119 species of the genus, *Thiobacillus thioparus*, was formalized as that isolated by
120 Starkey (1934) and deposited as ATCC 8158^T (Kelly and Harrison 1989). This strain
121 was widely used and was deposited as the type strain in various culture collections,
122 after passage through several laboratories. In addition, several new strains identified
123 physiologically as *T. thioparus* were deposited in culture collections.
124 Our aims were (1) to use 16S rRNA gene sequencing to assess whether examples
125 of the Starkey strain held in several major international collections were all correct,
126 given their different culture histories (Table 1); (2) to obtain a definitive reference 16S
127 rRNA gene sequence, against which other culture collections strains and new isolates
128 could be tested: the sequence currently available (M79426) is of relatively low quality;
129 and (3) to determine if newer isolates deposited as *T. thioparus* were in fact well-
130 founded examples of the species.
131 Additionally, we wished to obtain the16S rRNA gene sequence of the type strain
132 of *Halothiobacillus neapolitanus* NCIMB 8539^T, deposited by Parker as *Thiobacillus*
133 X (strain X44; Parker 1947, 1957; Parker and Prisk 1953; Parker and Jackson 1965).
134 The complete genome of a strain of *H. neapolitanus* (strain c2; ATCC 23641) was
135 available (NR_013422), and this strain is regarded as a derivative of the type strain X.
136 Its culture history prior to deposition was, however, significantly remote from the
137 original isolate: it was passed by Parker to P.A. Trudinger in the 1950s (Trudinger
138 1959, 1961, personal communication, 1966), by him to W. Vishniac in the USA, and

from Vishniac to D. White in the UK, who deposited it with the ATCC, using the code name "c2" employed in the taxonomic studies of White's group (Hutchinson et al. 1965, 1969): http://www.lgcstandards-atcc.org/LGCAdvancedCatalogueSearch/ProductDescription/tabid/1068/Default.aspx. Opportunity had thus existed for a change of properties, or contamination, compared to the original type strain. Our aims were to obtain a definitive reference sequence for the 16S rRNA gene of the type strain, and to validate the sequences for the well-studied strains of *H. neapolitanus* (ATCC 23641 and DSM 581). We also compared the 16S rRNA genes of the type strain of *Thiobacillus denitrificans* (NCIMB 9548^T) with those in the complete genome of strain ATCC 25259 (Beller et al. 2006).

Materials and Methods

The strains used (Table 1) were cultured from freeze-dried stocks from the ATCC, NCIMB, and the NITE Biological Resource Center (NBRC), or from live cultures on slopes (strains E6 and Tk-m) or in liquid culture (Starkey strain) from the DSMZ. Lyophilised strains were rehydrated in appropriate media (1 ml, 1 h), added to 10 ml medium and grown at 30 ºC for 5 days. Inocula (5 ml) from each culture were added to 50 ml medium in 250 ml flasks and shaken at 30 ºC. Media used for DSMZ and NCIMB cultures was a mineral medium (Boden et al. 2008; Kelly and Wood 1998) supplemented with 10 mM $Na_2S_2O_3$ for the initial 10 ml cultures, and with 3 mM $K_2S_4O_6$ for the main cultures. The use of tetrathionate avoided the problem of elemental sulfur precipitation from thiosulfate that is typical of *Thiobacillus thioparus* cultures (Kelly 1982; Kelly et al. 2005). Three of the ATCC strains were grown on ATCC 290 S6 medium (http://www.lgcstandards-atcc.org/Attachments/3616.pdf)

with tetrathionate instead of thiosulfate, but with thiosulfate for strain ATCC 23647, which preferred this substrate. Organisms were harvested for DNA extraction from the 50 ml cultures by centrifuging at 14,000 x g, 30 min, at 4 ºC. DNA was extracted from the organisms using the FastDNA® SPIN Kit for Soil (QBioGene, Cambridge, UK). PCR amplification of 16S rRNA genes was by standard procedures using the Lane 27f and 1492r primers (Lane et al. 1992). Sequencing (NCIMB and DSM strains) used DreamTaq and the BigDye terminator kit with the 27f, 341f and 1492r primers; and (ATCC strains) Platinum Taq and BigDye 3.1, with primers 27F, 770R, 704F and 1492R. Strains THI 111 and THI 115 were grown as described previously (Katayama-Fujimura et al. 1982; Katayama et al. 1992, 1998), and their 16S rRNA genes sequenced at the NBRC. Phylogenetic relationships were compared using the on-line NCBI blast algorithm tools (http://www.ncbi.nlm.nih.gov/blast), and construction of neighbour-joining distance trees (Fig. 1), using the CLUSTAL algorithm of MEGA 5. GenBank accession numbers for the new sequences of the *T. thioparus* strains are listed in Table 1.

Results and Discussion

Analysis of the 16S rRNA gene sequences of 12 strains of *Thiobacillus thioparus*

The 16S rRNA gene sequences for the four examples of the Starkey strain were essentially identical (Table 1, Fig. 1), and showed that the purity of these strains was maintained over a long period of culture in different laboratories and maintenance in the various culture collections. Similarly, strains Happold (h1), E6 (Smith and Kelly 1988) and Tk-m (Kanagawa and Mikami 1989) were confirmed as authentic strains of

189 *Thiobacillus thioparus* (Table 1, Fig. 1). Two strains (White 2K and strain THI 115)
190 showed lower sequence similarity to the type strain (Table 1), and appeared closer on
191 a phylogenetic tree to "*Thiobacillus sajanensis*" (Fig. 1). The Pankhurst T4 (p2)
192 strain formed a distinct clade with the GenBank sequences of the putative
193 *Thiobacillus thioparus* strains LV43 and API (Fig. 1). Two strains were clearly not *T.*
194 *thioparus*: the Pankhurst T1 and ParkerM strains showed only 83% and 85% identity
195 to the type strain (Table 1) and were indicated to be strains of *Halothiobacillus* and
196 *Thermithiobacillus* (Fig. 1). The 16S rRNA gene sequences of the type strains of *T.*
197 *thioparus* and *T. denitrificans* are about 98% similar, but none of the sequences
198 obtained was significantly more similar to *T. denitrificans* than was the type strain of
199 *T. thioparus* (Table 1, Fig. 1).
200 It is clear from our data, and the deposition history of the cultures, that the
201 definitive sequence for the 16S rRNA gene of the type strain of *Thiobacillus*
202 *thioparus* must be taken as that from ATCC 8158^T (HM173629). These data also
203 demonstrate that other culture collection examples of *Thiobacillus thioparus* should
204 be examined for phylogenetic identity to this type sequence. The situation identified
205 by us of some culture collection strains actually being examples of other species is
206 comparable to that revealed by an earlier study of culture collection strains of
207 *Paracoccus denitrificans* (Kelly et al. 2006; Rainey et al. 1999).
208
209 Status of some earlier database 16S rRNA gene sequences for *T. thioparus*
210
211 GenBank contains a sequence (M79426) for the type strain of *T. thioparus* (ATCC
212 8158^T), deposited in 1993 (Lane et al. 1992), and a sequence for strain LV43
213 (AF005628) from Movile Cave, Romania, isolated and analyzed by Vlasceanu et al.

(1997). These shared only 95.2% identity (1184/1244 aligned nucleotides) with each other, and the sequence for strain LV43 (AF005628) showed only 96.7% identity (1360/1406) to the newly determined sequence for the type strain (HM173629). This poor match was due in part to the presence of 20 unidentified (N) nucleotides in the sequence, but if the 'N' positions were replaced with the corresponding nucleotides from the HM173629 sequence, the identity to HM173629 still only became 97.9%. Comparison of this modified LV43 sequence with M79426 (which also contains 3 'N' positions) still showed 96.5%, suggesting a low relationship between these strains at the species level. The M79426 sequence showed 98.6% identity (1226/1244) to the new sequence for the type strain (HM173629), confirming that M79426 can no longer be accepted as representing the 16S rRNA gene of the type strain of *T. thioparus*. Strain LV43 has unfortunately been lost from culture (B. Kinkle, personal communication) so cannot be re-examined, but even the poor sequence available indicates it to have been a strain of *T. thioparus*, and to share a closer identity to the Pankhurst T4 strain (Fig. 1) and to the partial sequence for strain API, with which it shares 97-98% identity. The Pankhurst T4 strain produced little tetrathionate when cultured on thiosulfate, and increased acidity of the medium to about pH 4.4 during growth on 40 mM thiosulfate, which is typical of *T. thioparus*. We concluded that the T4 strain and strains White, LV43 and API were all very similar to *T. thioparus*, and insufficiently different from the type strain for revision of their status without further analysis.

Reassignment of *T. thioparus* strain Pankhurst T1 (NCIMB 8454) as a strain of *Halothiobacillus neapolitanus*

239 This strain, originally deposited as *Thiobacillus thioparus*, was isolated from a
240 thiosulfate-oxidizing mixed culture (Pankhurst 1964). Our 16S rRNA gene sequence
241 analysis indicated it to be a strain of *Halothiobacillus neapolitanus* (Table 1, Fig. 1),
242 which has resulted in the NCIMB reclassifying it as a strain of that species. Its
243 physiological properties are consistent with those reported for the type species
244 (NCIMB 8539^T). Pankhurst (1964) showed it to produce large amounts of
245 tetrathionate from thiosulfate, amounting to about 66% of the thiosulfate-sulfur after
246 three days, along with some formation of trithionate, but subsequently oxidized the
247 tetrathionate to sulfate, with an increase in acidity (Pankhurst 1964). These properties
248 are typical of *H. neapolitanus*, which rapidly produces tetrathionate (and trithionate)
249 both in cell suspensions and when grown in batch culture (Kelly 2008; Trudinger
250 1959, 1964). Tetrathionate production is effected by a thiosulfate-oxidizing enzyme
251 (Trudinger 1961), and the amount produced is strongly affected by environmental
252 conditions, in extreme cases resulting in virtually quantitative conversion of
253 thiosulfate to tetrathionate (Kelly 2008; Trudinger 1964). Typically, the Pankhurst T1
254 strain quantitatively converted 40 mM thiosulfate to sulfate with a drop in pH from
255 about pH 6.8 to pH 3.2, which is typical of *H. neapolitanus*.
256
257 Definitive 16S rRNA gene sequence for the type strain of *Halothiobacillus*
258 *neapolitanus* strain X (NCIMB 8539^T) and comparison with existing database
259 sequences
260
261 The only sequence available on GenBank for this strain between 1993 and 2011 was
262 AH001797, which consisted of three segments, totalling only 903 bp, of which 11
263 were unidentified nucleotides. The nucleotide sequence of the authentic type strain X

(NCIMB 8539) was determined in order to ensure a definitive reference sequence was available (JF416645; 1379 bp). Comparison, using the BLASTN algorithm, of the sequences JF416645 and AH001797 showed a cumulative similarity of only 97.4% (876/899 nucleotides), reflecting the relatively poor quality of the old sequence. The complete genome of *H. neapolitanus* strain c2 (ATCC 23641) became available in 2011 (NC_013422). This strain was a derivative of the original type strain X, and contained two genes for the 16S rRNA (loci Hneap_R0016 and Hneap_R0052), each comprising 1524 bp. These differed from each other only by one nucleotide (G instead of A at position 314 in Hneap_R0016). The newly-determined type strain sequence showed similarities to Hneap_R0016 and Hneap_R0052 of 1379/1379 and 1378/1379 nucleotides, with the mismatch to Hneap_R0052 being G at position 314. The JF416645 sequence was thus identical to the Hneap_R0016 gene of strain c2. Comparison of the gene sequences of another authentic strain of *H. neapolitanus* (strain C; DSM 581, AF173169) and of the newly-designated strain Pankhurst T1 (NCIMB 8454; HM173632) with that of strain X and genes Hneap_R0016 and Hneap_R0052 of strain c2 gave sequence identities of 99.8-100%, and showed both to have expressed the Hneap_R0052 gene. The GenBank database currently contains sequences for five other putative strains of *H. neapolitanus* (EU871645, AB308268, HQ693550, EU591537, AY686547), all of which are correct when compared to the new type strain sequence. Three of these sequences are long (1259, 1399 and 1470 bp) and show 99.3-99.6% identity to JF416645, and all three were examples of the Hneap_R0052 gene. The two shorter sequences (555 and 991 bp) shared 99.1 and 99.3% identity with the JF416645, Hneap_R0016 and Hneap_R0052 sequences, but their coverage did not include the nucleotide at the 314 position of Hneap_R0016. The database contains a number of other nearly complete sequences attributed to

289 strains of "*Thiobacillus* sp." and "*Halothiobacillus* sp." (e.g. AY487255, GU013549,
290 AY096035, EU912480, EF397577, FM992406), which showed 99.0-99.8% identity
291 to the type strain sequence JF4156645, and all were examples of the Hneap_R0052
292 gene.
293 The sequence of the gene from *H. neapolitanus* strain X has been adopted as the
294 reference sequence in the *List of Prokaryotic Names with Standing in Nomenclature*
295 (http://www.bacterio.cict.fr/h/halothiobacillus.html).
296
297 Reassignment of *T. thioparus* strain ParkerM (NCIMB 8349) as a strain of
298 *Thermithiobacillus*
299
300 This strain (M79) was one of several similar bacteria isolated from a corroded
301 concrete sewer, and quantitatively converted thiosulfate to tetrathionate, which was
302 apparently not oxidized to sulfate (Parker 1947; Parker and Prisk 1953). This led
303 Parker and Prisk (1953) to conclude that these strains were not actually
304 chemolithoautotrophic thiobacilli, but were more likely to be related to the
305 heterotrophic "*Thiobacillus trautweinii*" described by Trautwein (1921), which was
306 later reclassified as *Pseudomonas* sp. NCIMB 9549. Strain ParkerM was, however,
307 thought to be a strain of *T. thioparus* by K.R. Butlin and J.R. Postgate, who deposited
308 it as NCIMB 8349 in 1959 (http://www.ncimb.com/results.php?parent=culture). As a
309 result of our 16S rRNA gene sequence analysis strain ParkerM appeared to be only
310 the second strain of *Thermithiobacillus* in a culture collection, and has been
311 reclassified as *Thermithiobacillus* sp. by the NCIMB (Table 1, Fig. 1). Its
312 physiological properties are very similar to those reported for the type strain, which
313 quantitatively converted thiosulfate to tetrathionate before further oxidation to sulfate

(Wood and Kelly 1985, 1986). Under the conditions employed by Parker and Prisk (1953), cultures on 40 mM thiosulfate rose from pH 6.6 to pH 7.5-7.8, with conversion of about 92% of the thiosulfate consumed to tetrathionate. Under our more favourable culture conditions, oxidation of tetrathionate to sulfate proceeded with a consequent increase in acidity to about pH 5.2, which is the same as the minimum of about pH 5.2 reported for the type strain provided with 10 mM tetrathionate or 20 mM thiosulfate (Wood and Kelly 1985, 1986).

Taxonomic status of the available strains of *Thiobacillus denitrificans*

Since this species was established by Beijerinck (1904), numerous strains described as *T. denitrificans* have been used in biochemical and commercial studies, but no authentic strain was available at the time Hutchinson et al. (1967, 1969) began their taxonomic studies, and currently only six distinct strains appear to be held in international culture collections. Three of these were isolated by Hutchinson et al. (1967; NCIMB 9546, 9547 and 9548^T), one each came from Texas soil (ATCC 25259; Taylor et al. 1971), Senegalese mud (DSM 807; Baldensperger and Garcia 1975), and pond water (DSM 739; H. Hippe). The type strain (NCIMB 9548^T; Kelly and Wood 2000b) is held by at least six collections (NCIMB, ATCC, DSMZ, CIP, JCM, and BCRC), and was used in the phylogenetic studies of Lane et al. (1985), and by Justin and Kelly (1978). The six available strains have not been the subject of detailed comparisons using modern molecular methods, except for strain ATCC 25259, for which the complete genome is available (NC_007404; Beller et al. 2006). This genome contains two identical copies of the 16S rRNA gene, but these show only 97.6% sequence identity to that of the type strain. This is comparable to the

difference between the distinct species, *T. denitrificans* and *T. thioparus* (Table 1), which raises the possibility that the type strain and ATCC 25259 may be examples of distinct nitrate-reducing species. We have included comparative 16S rRNA gene sequence similarities between NC_007404 and the twelve species in Table 1, to show that the similarities are only slightly different from those seen with the type species. Running BLASTN searches of the GenBank database with the AJ243144 (type) and the Tbd_R0009 16S rRNA gene of ATCC 25259 showed the type strain sequence to share 98.8-99.5% sequence identity to *Thiobacillus* strain NB457 (HQ851052) and two uncultured clones (HQ015463, FN436148), but the ATCC sequence showed only 97.2-97.6% identity to these. Conversely, the ATCC sequence showed 99.3 and 98.8% identity to a different uncultured clone (HQ132467 and to *Thiobacillus* strain ME16 (EU546130), which were only 97.3% and 98.1% identical to the type sequence. All other BLAST 'hits' to sequences on the database were 97.2-98.2% for both strains, consistent with both types being distinct, and occurring in the natural environment. These comparisons show that ATCC 25259 had no nearer neighbours with validly published names than *T. thioparus* and *T. denitrificans* (type strain), but may not be an example of either. This indicated that detailed molecular and physiological comparisons of these strains, and with others in the culture collections and laboratories is needed, as there could be as great a genetic diversity among physiologically similar strains that are currently assigned to *T. denitrificans* as was established with physiologically similar iron-oxidizing strains regarded as *Acidithiobacillus ferrooxidans* (Amouric et al. 2011; Harrison 1982; Karavaiko et al. 2003; Kelly and Harrison 1989; Waltenbury et al. 2005).

Conclusions

Our study has confirmed the species affiliations of a number of phenotypically similar strains of *Thiobacillus thioparus*, but has reassigned two strains originally defined solely by their physiological properties as *T. thioparus* to two alternative genera. Based on 16S rRNA gene sequences, these alternative genera, *Thermithiobacillus* and *Halothiobacillus*, are phylogenetically more remote from *Thiobacillus* than *Thiobacillus* is itself, for example, from *Neisseria* or *Escherichia* (Kelly et al. 2005). The phylogenetic tree (Fig. 1) showed *Thermithiobacillus* to cluster more closely with *Acidithiobacillus* than with the other genera. Recently, in a multiprotein family comparison of the genomes of 104 *Gammaproteobacteria*, the *Acidithiobacillales* were concluded to comprise a group that was distinct from both the *Gammaproteobacteria* and the *Betaproteobacteria*, probably having diverged after the formation of the *Alphaproteobacteria* but before the *Gammaproteobacteria-Betaproteobacteria* split (Williams et al. 2010). This is interesting because *Acidithiobacillus* was originally placed in the *Betaproteobacteria* (Lane et al. 1992), apparently lying near the *Gammaproteobacteria-Betaproteobacteria* root, but was assigned to the *Gammaproteobacteria* by Kelly and Wood (2000a) in their revision of the *Thiobacillus* genus. It may indicate that *Thermithiobacillus* also falls outside the *Gammaproteobacteria*, but this can only be resolved when the genome of *Th. tepidarius* becomes available.

The finding that the 16S rRNA gene sequence of the type strain of *T. denitrificans* differs significantly from that of the ATCC 25259 strain shows the importance of using the sequences of the type strains, as provided in the *List of Prokaryotic Names with Standing in Nomenclature* (http://www.bacterio.cict.fr/h/halothiobacillus.html).

It has recently been argued that 16S rRNA gene sequence comparisons are inadequate for the discrimination of individual species, as the genes are too highly conserved (Staley 2006, 2009). The 2nd edition of *Bergey's Manual of Systematic Bacteriology* (2005) was, however, constructed on the phylogenetic framework provided by 16S rRNA gene sequence analysis, down to the species level. Small variations in this highly conserved macromolecule have proved an immensely valuable and largely reliable tool in species as well as genus discrimination, when used in conjunction with phenotypic properties. Advances in molecular methods now make 16S rRNA data part of an arsenal of phylogenetic tools, coupled with DNA hybridization and multiple locus sequence analysis, to enable a holistic approach to taxonomy. For discrimination of phenotypically similar genera, 16S rRNA gene sequence analysis is still the most rapid of the powerful and reliable taxonomic tools available for species separation. It potentially provides a means by which commercial culture collections can assess the identities of stock cultures deposited before the widespread use of molecular methods in taxonomy, and has proved useful in the cases described here, and in the earlier reassessment of some *Paracoccus* strains in culture collections (Kelly et al. 2006).

Acknowledgement

We are grateful to Dr Ann Wood for constructive comments on the manuscript.

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Legend to Figure 1

Fig. 1 Neighbour-joining distance tree, based on nearly complete 16S rRNA gene sequences, aligned using the CLUSTAL algorithm of MEGA 5, showing the position of 12 strains originally received as *Thiobacillus thioparus*, compared to their phylogenetic neighbours. Two of these were reassigned to different genera: NCIMB 8454 to *Halothiobacillus neapolitanus*, and NCIMB 8349 to *Thermithiobacillus* as a result of this study. The reference sequence for *Halothiobacillus neapolitanus* NCIMB 8539^{T} was also newly obtained in this study, for comparison with that from the complete genome of strain c2 (ATCC 23641; NC_013422), which is a descendent of the type strain X (NCIMB 8539^{T}). The sequence previously available for the type strain (AH001797) is only partial and is segmented. The sequence of *Paracoccus denitrificans* ATCC 17741^{T} (*Alphaproteobacteria*) was used as outgroup. Strains newly sequenced in this study are shown in **bold** font. Numbers on branch nodes are bootstrap values above 70% (from 1000 resamplings). Bar, one estimated substitution per 100 base positions.